CHAPTER NINE

Summary and

Discussion

Leukemias are characterized by a defect in the regulation of proliferation and differentiation of immature malignant progenitors resulting in the replacement of the normal hematopoietic precursor cells by leukemic blast cells and, ultimately, in bone marrow failure (see 1.2.2). The treatment of leukemic patients consists of intensive induction and consolidation chemotherapy resulting in an overall survival of 30-40% (Zittoun *et al.*, 1995). During the last years different approaches have been applied to increase the dose-intensification and subsequently to enlarge the number of long-term survivors (Burnett and Eden, 1997). Conditioning therapy with high-dose chemotherapy followed by allogeneic- or autologous bone marrow cell transplantation are the most frequently applied methods. Autologous stem cell transplantation was first successfully employed to cure patients with lymphoma in the late 1970's (Appelbaum *et al.*, 1978) and its use became widespread in the 1980's (Gale and Butturini, 1989; Stuart, 1993). Today, the number of autologous bone marrow transplantations is surpassing that of allogeneic transplantation as potential curative treatment modality for several kinds of hematologic malignancies (Gale and Butterini, 1989; Santos, 1990; Stuart, 1993). Also in the treatment of other than hematological malignancies (autologous) bone marrow transplantation is widely used (Horrowitz *et al.*, 1993; Lazarus, 1993; Matthay *et al.*, 1993; Lotz *et al.*, 1994; Shpall *et al.*, 1994; Friedrich, 1996; Joensuu, 1996; Meisenberg *et al.*, 1997; Moreb *et al.*, 1997). A variety of purging modalities have been applied to the treatment of hematopoietic malignancies in order to deplete residual malignant cells in autologous bone marrow (see 1.3). The aim of the experiments presented in this thesis was to explore the potential of hyperthermia as a powerful purging modality by a detailed analysis of the hyperthermic sensitivity of murine and human normal and leukemic subsets.

9.1 Conclusions from the experiments.

At the start of the studies described in this thesis, the most primitive subset studied so far was the murine CFU-S. No information on the heat sensitivity of the totipotent hematopoietic stem cells responsible for the long-term engraftment was available. A wide variation in the outcome of hyperthermic treatments on the progenitor subsets was reported in the literature. This might be due to the differences in experimental approaches. In *Chapter 2* the heat induced cytotoxicity on the normal hematopoietic subsets CFU-S-8 and CFU-S-12 is reported. From these experiments it became clear that the hematopoietic subsets differ in heat sensitivity depending on the source of the two subsets, i.e. bone marrow from normal mice and bone marrow and spleen cells from anemic mice. The CFU-S-12 appeared to be more resistant to heat compared with the CFU-S-8 in normal bone marrow. The main conclusion was that the proliferative activity and hyperthermic sensitivity of the CFU-S subsets are interrelated. For instance, if the proliferative activity of the subsets are comparable as is the case in

bone marrow or spleen from anemic mice, the heat sensitivity of the two subsets do not differ significantly. This indicates that the intrinsic sensitivity of both subsets to heat is comparable but that the differences in proliferative activity are responsible for the detected differences in heat sensitivity.

By using purified CFU-S-12 from bone marrow of normal and anemic mice it was demonstrated that the observed heat effects were a direct effect on the hematopoietic subsets (*Chapter 3*) and not due to indirect actions of released substances from heat-damaged cells in the environment. It could also be excluded that the heat treatment resulted in a selection of macroscopically detectable colonies. The composition of the spleen colonies was unchanged as demonstrated by a comparable morphology of the spleen colonies before and after the heat treatment.

In *Chapter 4* the hyperthermic sensitivity of the relevant clonogenic subsets in murine bone marrow (MRA, CFU-S-12, CFU-S-8, CFU-GM, BFU-E and CFU-E) was documented. In this study the supposed relationship between stem cell hierarchy and heat sensitivity (Chapter 2 and 3) was confirmed. The heat sensitivity increased with increased maturation, i.e. the primitive hematopoietic stem cell (MRA) appears to be most heat resistant subset within the hematopoietic stem cell compartment. Because proliferation increases with maturation, hyperthermic sensitivity increases.

In order to gain knowledge about the effect of hyperthermia on the hematopoietic stem cell with long-term repopulating ability (LTRA) an *in vivo* assay using a congenic mouse strain was used. This is reported in *Chapter 5*. Comparison with the hyperthermic sensitivity of the hematopoietic subsets, as demonstrated in the previous chapters, revealed that LTRA cells are less sensitive to hyperthermic treatment than the committed progenitor subsets again confirming the dependency of heat-induced cell kill on the proliferative activity and hence hierarchical position. This relationship is illustrated in Figure 9.1.

In *Chapter 6*, the impact of hyperthermic treatment on normal and leukemic cells is compared. It could be demonstrated that at a heat dose resulting in a 4-log depletion of leukemic cells (L1210), the primitive hematopoietic stem cell (MRA) is killed by less than 1-log. The differences in heat sensitivity between the leukemic cells and the normal subset MRA were quite large. However, a 2-log cell kill was observed for the progenitor subset CFU-GM. Because these cells are responsible for short-term engraftment after bone marrow transplantation, it will be appreciated that an increase in the ratio between the surviving fractions of leukemic cells and normal progenitor cells (“therapeutic gain factor”) will enlarge the possible clinical application of

hyperthermia as a purging modality. The therapeutic gain factor can be modulated by decreasing the heat sensitivity of the normal hematopoietic subsets, increasing the heat sensitivity of the leukemic cells, or by achieving both effects. From the presented data it can be concluded that the leukemic cells already possess a relative high heat sensitivity and therefore a decrease of the heat-induced cell kill of the normal hematopoietic subsets seems to be a logical approach. In this chapter it is shown that the tetrapeptide AcSDKP (Goralatide) provided a selective inhibitory effect on the

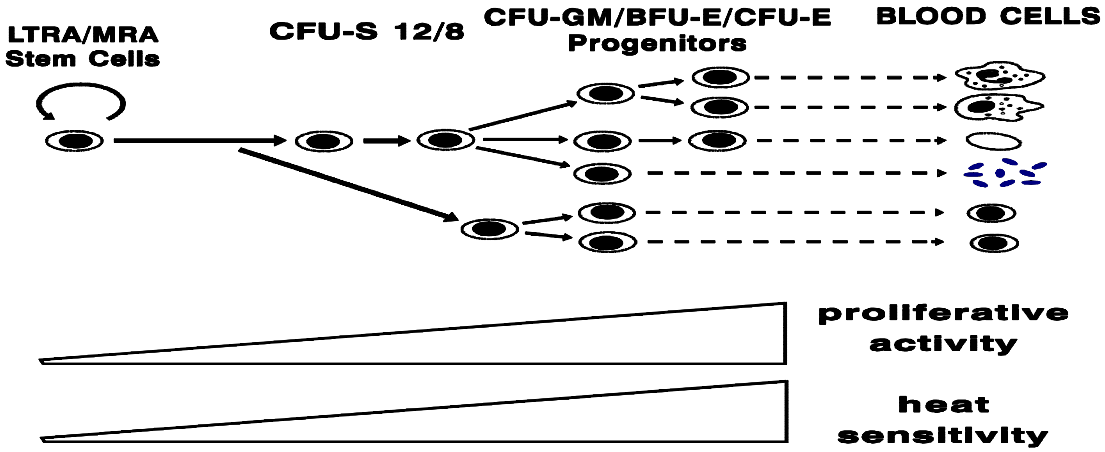

Figure 9.1. *Relationship between stem cell hierarchy and heat sensitivity of normal subsets within the hematopoietic stem cell compartment.*

proliferative activity of normal hematopoietic progenitor cells. Thus pretreatment with Goralatide resulted in a thermoprotective effect on the normal progenitor cells and increased the therapeutic gain factor to $\geq$ 500. So, hyperthermic treatment at 43°C has significant potential as a purging modality.

The disadvantage of leukemic cell lines is that they consist of a homogeneous cell population, not representative for the clinical situation. Therefore, the heat sensitivity of an *in vivo* murine acute myeloid leukemia model was studied (*Chapter 7*). Originally, this myeloid leukemia was induced by X-ray exposure (Mole *et al.*, 1983). Serial passages at low cell dose were used to conserve the primary kinetics and morphological properties of the leukemia (Hepburn *et al*., 1987). The effect of *in vitro* heat treatments on the murine myeloid leukemia was studied in mice by determining the development of leukemia after inoculation of the treated sample. In this way,

information was obtained about the effects on the leukemic stem cell responsible for the leukemic development. The heat-induced cell killing effects were comparable with that on L1210 cells presented in Chapter 6. To determine the heat sensitivity of the entire heterogeneous leukemic stem cell compartment in this model and to compare it with the normal hematopoietic stem cell compartment, experiments were performed using the CAFC-assay. The data obtained with the conventional clonogenic assays for normal hematopoietic stem cells were confirmed with this assay, i.e. the early appearing cobblestone areas (= progenitor subsets) are far more sensitive to a heat treatment than the late appearing cobblestone areas (= primitive stem cells). For the leukemic subsets the differences in heat sensitivity are less pronounced despite the differences in proliferative activity of early and late appearing cobblestone areas. Based on these experimental data, a hyperthermic purging protocol was developed and tested in vivo. Remission bone marrow was simulated by adding 5% leukemic cells to a normal bone marrow cell suspension. This cell suspension was either left untreated or treated with a heat dose with and without Goralatide pretreatment before transplantation into lethally irradiated mice. Mice transplanted with the heat treated marrow died before day 15 due to aplasia. The pretreatment with Goralatide resulted in survival of the recipients (> 200 days) without any sign of leukemia. This *in vivo* approach once more demonstrated the feasibility of hyperthermia as purging modality.

Extrapolation of murine to human needs careful consideration and therefore the research was continued using human bone marrow (*Chapter 8*). A number of subsets in normal and leukemic bone marrow samples were tested for their sensitivity to a heat treatment at 43°C. It was found that the therapeutic effect of the hyperthermic purging protocol is even more pronounced in human bone marrow compared with the murine data. At a heat dose which eliminates leukemic cells by 5-log, only a half log depletion in normal CFU-GM was observed. Again a selective protection of the normal hematopoietic progenitor cells from heat damage could be demonstrated after a pretreatment with Goralatide. Without the Goralatide pretreatment the therapeutic gain factor was 1000 which is clearly above the defined minimum level (see 1.3). Whether this therapeutic effect also holds for stem cells needs further investigation (see Future experiments). However, based on the murine data it is to be expected that the human totipotent stem cells demonstrate a lower heat sensitivity than the progenitor cells. Changes in heat sensitivity between quiescent and active proliferating leukemic subsets in the murine acute myeloid leukemia model are far less pronounced. If the same is true for human leukemic subsets an increase in the therapeutic effect seems certain.

Possible mechanisms for the detected differences in heat sensitivity between normal and leukemic subsets

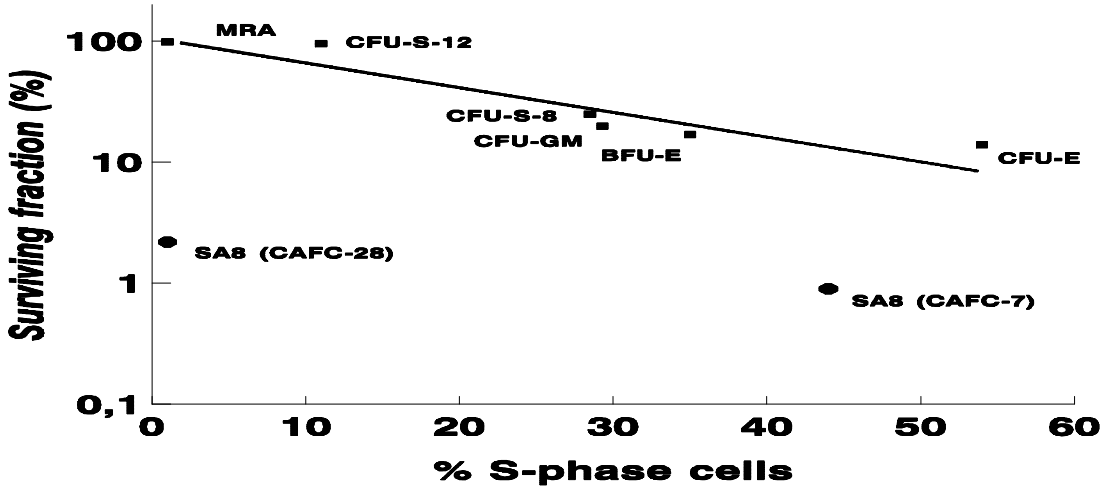

Figure 9.2. *Relation between the proliferative activity and heat sensitivity of normal hematopoietic and leukemic subsets.*

It remains unclear why the heat sensitivity of normal hematopoietic subsets and their leukemic counterparts differ as much as they do. Although the proliferative activity is a major determining factor for heat sensitivity, the observed differences between normal and leukemic subsets cannot be explained by this cell-cycle feature alone. The data on the heat-induced cell kill for both resting and active cycling leukemic subsets do not fit the regression line for the comparison between the proliferative activity and thermal sensitivity of the normal hematopoietic subsets as is shown in Chapter 4 and in Figure 9.2.

In contrast to the actively proliferating murine progenitor cells, a shoulder region in the survival curve is observed for the normal quiescent hematopoietic subsets (Chapter 4). This can be explained by a threshold for accumulation of heat-induced damage before lethality becomes manifest in these quiescent subsets. Interestingly, the same phenomenon is observed in human bone marrow. The survival curves for the human hematopoietic progenitors also fail to demonstrate a shoulder region (Chapter 8). However, if the proliferative activity of these human subsets is reduced to very low levels by the pretreatment with Goralatide a shoulder region in the survival curves can be observed. A shoulder region could not be constructed for the leukemic stem cell, indicative of the absence of a threshold for the accumulation of heat-induced damage (Chapter 6 and 7). Possible differences in the amount of heat sensitive proteins, e.g. in the membranes, between normal and leukemic cells might be a factor in the observed

differences in heat sensitivity. However, the lack of purification techniques for leukemic stem cells frustrates studies in this area.

For the same reason, studies of the possible role of heat shock proteins (HSPs) in heat-induced cell killing of hematopoietic and leukemic cells are hampered. These type of proteins are believed to play a role in heat sensitivity/resistance. Most heat shock proteins share the ability to bind to polypeptides to assist in proper protein folding and transport of proteins across cellular membranes. Chaperone functions may explain their protective function against heat-induced protein denaturation (Ellis and Van Der Vies, 1991; Stege *et al*., 1994b). For instance, heat shock proteins may bind to partial heat-induced denatured proteins and protects them against aggregation and/or assist in refolding and/or in removal of irreversibly damaged proteins. The HSP70 family contains at least four members (Welch, 1990) of which in view of this thesis HSP73 is the most relevant. HSP73 has been found to be associated with heat resistance (Laszlo, 1988). Some interesting observations were made by Banerji *et al*. (1987) who showed that HSP73 is temporally expressed during maturation of avian erythroid cells. The highest levels of this heat shock protein are detected in primitive red cells and during maturation the levels of HSP73 synthesis decline. If this hierarchical related decline in HSP73 synthesis also would exist in the hematopoietic stem cell compartment, this would be in accordance with the claimed heat resistance function of HSP73 and the detected decrease in heat sensitivity within the hematopoietic stem cell compartment. For another heat shock protein, HSP90, alterations in the synthesis upon differentiation induction of granuloid cells is observed (Yufu *et al*., 1989). Although the cell cycle status is the main determining factor for the differences in heat sensitivity of the hematopoietic stem cell compartment, a role for heat shock proteins cannot be excluded. Studies in the human leukemic cell line HL-60 revealed very low levels of constitutive HSP70 and HSP90 (Yufu *et al*., 1990). In contrast, Chant *et al*. (1995) found no differences in constitutive levels of HSP60, 70 and 90 between normal human mononuclear cells and chronic myeloid leukemic cells while the expression of all three heat shock proteins was significantly higher in acute myeloid leukemic cells compared to normal mononuclear cells. The discrepance in constitutive levels of heat shock proteins between these two type of leukemia might be related to the differentiation state of these leukemic cells and complicate the interpretation of the thermoprotective effect of HSP70. However, we must realize that these studies were performed on partially purified cell populations. As long as no purified leukemia-initiating stem cells are available, no unequivocal answer on the role of heat shock proteins in the observed differences in heat sensitivity can be given.

The main conclusions can be summarized as follows:

a) the intrinsic heat sensitivity of the different subsets in the hematopoietic stem cell compartment is comparable.
b) the hyperthermic sensitivity and proliferative activity of the different subsets are interrelated and responsible for the observed differences in sensitivity to heat treatments.
c) the heat sensitivity of normal hematopoietic progenitor cells differ significantly from that of leukemic cells and cell lines.
d) the heat sensitivity of normal hematopoietic subsets can selectively be protected by a pretreatment with Goralatide.
e) the therapeutic gain factor of a hyperthermic purging protocol (90' at 43°C) fulfills the clinical requirements of a feasible purging modality.

Thus, the results of the experiments described in this thesis strengthen the case of using hyperthermia as a purging agent in autologous stem cell transplantation protocols. In future experiments we will try to increase the therapeutic efficacy of hyperthermia as purging modality by the use of a specific heat protector of normal bone marrow subsets and a specific heat sensitizer of leukemic stem cells. We would also like to generate tools to evaluate the potential to introduce hyperthermia as a purging modality in a clinical trial. These continuing studies might reveal optimal treatment conditions resulting in a highly efficient, safe and low cost purging protocol that can directly be implemented in clinically autologous stem cell transplantations and which reduces the likelihood of relapse.

9.2 Recent developments in autologous transplantation

Bone marrow is most commonly the source of hematopoietic stem and progenitor cells and was also used in the experiments described in this thesis. However, the use of peripheral blood cell transplantation for the treatment of a variety of hematologic and oncologic malignancies has been expanded rapidly over the last decade (Ahmed *et al.*, 1994; Bodine, 1995; Mangan, 1995; Kamble *et al.*, 1997; Reiffers *et al.*, 1996; Shpall *et al.*, 1997; To *et al.*, 1997). Because peripheral blood cell grafts may contain limited numbers of hematopoietic stem cells, numerous approaches to expand the hematopoietic subsets have been applied, e.g. *in vivo* after high-dose chemotherapy (Spangrude *et al.*, 1995; Diaz-Mediavilla *et al.*, 1996; Morton *et al.*, 1997) or *in vitro* using growth factors (Muench *et al.*, 1994; Brugger *et al.*, 1995; Pettengell, 1995; Ho *et al.*, 1996; Imamura *et al.*, 1996). Umbilical cord blood cells also have potential in reconstituting the hematopoietic system (Cardoso *et al.*, 1993; Almici *et al.*, 1995; De Bruyn *et al.*, 1997). The rationale for using blood cell grafts is firstly, that in such a transplantation protocol hematopoietic subsets are easy to collect by means of leukapheresis, thus avoiding the need for patients to undergo general

anesthesia; secondly, peripheral blood cell transplantation can be offered to patients who are not candidates for bone marrow harvest because of marrow fibrosis. Thirdly, it is generally true that hematopoietic recovery following peripheral blood cell transplantation is significantly quicker than usually observed after bone marrow transplantation (Harousseau *et al.*, 1995; Indovina *et al.*, 1995; Brice *et al.*, 1996; Hartmann *et al.*, 1997) and this could lead to a decrease in the morbidity and mortality of the transplantation procedure. However, other reports suggest an impaired capacity to support long-term engraftment (Dreger *et al.*, 1995; Klumpp, 1995; Bentley *et al.*, 1997; Chang *et al.*, 1997). This would imply a difference in composition and/or engraftment quality between a peripheral blood cell and bone marrow graft (Eaves, 1993; Hogge *et al.*, 1993; Janssen, 1993; Tong *et al.*, 1994; Wickenhauser *et al.*, 1995; Yamamoto *et al.*, 1996). Finally, it has been hypothesized that peripheral blood cell grafts are less contaminated by residual tumor cells but several reports demonstrate that together with the mobilization of normal progenitor cells during chemotherapy treatment an increase in tumor cells into peripheral blood is observed (Brugger *et al.*, 1994; Scheding *et al.*, 1994; Gee, 1995; Moolten, 1995; Brugger *et al.*, 1996). Since the sources of hematopoietic subsets for transplantation differ today, the terminology “stem cell transplantation” is now more accurate. Nevertheless, whatever the source of autologous stem cells, contamination with malignant cells is likely.

9.3 To purge or not to purge.....is that the question?

In patients who relapse after autologous stem cell transplantation the recurrence of the disease might derive from the endogenous hematopoietic system caused by limiting efficacy of the conditioning regimen or it might occur from malignant cells present in the graft (bone marrow or peripheral blood) at the time of transplantation, or both. A variety of methods have been used to eliminate or “purge” neoplastic cells from remission marrow suspensions prior to cryopreservation (see 1.3). However, the value of purging in the clinical outcome of autologous stem cell transplantation has been a matter of debate for many years (Armitage, 1994; Kvalheim, 1996; Löwenberg and Voogt, 1996).

For instance, a hypothetical model for estimating the probability of leukemia arising from an autograft was developed by Hagenbeek’s group (Hagenbeek and Martens, 1985; Schultz *et al.*, 1989). An index termed ED_{50} was introduced which defines the number of malignant cells required to induce leukemia in 50% of recipients. They posit that the quantity of inoculated cells is equivalent to the number of ED_{50} units and that one of two possibilities of equal probability takes place: either the unit disappears or clonogenic cells propagate. By extrapolation from the Brown Norway rat model of acute myeloid leukemia, they postulate that the ED_{50} for patients is likely to

be 10^4 clonogenic cells. Based on this assumption the authors estimated the contribution of leukemic cells present in the autograft to recurrence of the disease. They state that an average autograft contains $\sim 1.5 \times 10^{10}$ nucleated cells including 1.5×10^6 leukemic cells; i.e., a 4-log reduction after the induction therapy. Furthermore, they suggest that cryopreservation depletes the leukemic cells by another 2-logs and that only 1% of the leukemic cells are truly clonogenic. Hence, in their estimation an autograft contains about 150 clonogenic cells suggesting that the probability of leukemia arising from the transplant will be 1% or less and led to the conclusion that purging is not necessary.

I would like to make some critical remarks on this theoretical approach. Firstly, if a 4-log reduction of leukemic cells in a patient can be accomplished, one might question whether an autologous bone marrow transplant should be the primary treatment for that patient. After the induction of remission, maintenance therapy might be sufficient for extended leukemia free survival. The suggested depletion of leukemic cells after induction of remission might be an overestimation of the situation in clinical practice. The clinical status of patients receiving a bone marrow transplant, is referred to as being in complete remission. Here reiterating the definition of the term “complete remission” is appropriate. The general consensus is that a contamination of $\leq$ 5% leukemic blasts in the bone marrow is considered as a bone marrow in complete remission (see also Figure 1.1). Thus, a 2-log reduction in leukemic cells is already below the threshold for the qualification of a complete remission. High-risk patients might be those patients in which the induction therapy results in a reduction of the leukemic burden by about 2-logs and this limited depletion might be one of the causes for their poor prognosis. Especially in these patients a high-dose conditioning therapy followed by bone marrow transplantation is inevitable. Based on the calculations in the above cited studies, the leukemic burden in the graft will be 1.5×10^8 cells and hence a purging effect of 4-log is necessary to meet the requirements of a low probability for recurrence from the graft. This level of depletion is in accordance with the general consensus that a 4-log depletion of the leukemic burden in an autograft is required for an efficient purging modality (Trickett, 1987; Keating, 1991; Gribben and Nadler, 1993; Ball and Rybka, 1993).

Secondly, it should be emphasized that in this theoretical approach, cryopreservation was part of the overall protocol. Thus, their theory is based on the inclusion of a purging technique, although only a 2-log depletion of leukemic cells is assumed. The conclusion that the application of purging techniques are irrelevant to the clinical outcome of the transplanted patient cannot be made on the assumption and calculations made in these studies.

In another study, questioning the positive role of purging, the results of syngeneic transplantations for acute myeloid leukemia in first remission for a series of 44 patients was reported (Horowitz *et al.*, 1989). The authors found a leukemia free survival (defined as survival without evidence for leukemia over a period of at least one year) of 39% compared to 34% for unpurged autologous transplanted patients. Based on these data the authors concluded that relapses are unlikely to arise from leukemic clones in the autograft. I would like to add that the percentage of leukemia free survival reported in this study is rather low compared to other data (Gorin *et al.*, 1990, 1991; Ball and Rybka, 1993; Stuart, 1993). This low level might be the result of a suboptimal conditioning therapy thereby increasing the risk of recurrence of the disease predominantly from residual malignant cells in the patient.

By contrast, Gorin *et al.* (1990, 1991) reported a positive correlation between marrow purging with mafosfamide and relapse in combined studies of the European Bone Marrow Transplantation Group. More than 900 patients with acute myeloid leukemia in first remission were transplanted with autologous bone marrow. Most of the patients (629) received unpurged bone marrow. A total of 269 patients has their marrow purged with mafosfamide. The median follow-up was 28 months. The superior results of purging were demonstrated by a decrease in the probability of relapse from 60 ± 6 % for patients receiving purged to 16 ± 6 % for those receiving unpurged bone marrow, depending on the conditioning therapy. The lower relapse rate was improved when the dose of mafosfamide was adjusted to the individual sensitivity of progenitor cells.

The most convincing evidence for the rationale for purging comes from studies of Brenner and associates. These studies (Rill *et al.*, 1992; Brenner *et al.*, 1993; Rill *et al.*, 1994), using gene marked grafts, have clearly demonstrated that the leukemic relapse indeed originates in part from the autograft . Both the transferred genetic marker and a tumor-specific marker were determined in the same cell population to investigate whether residual leukemic cells in the autologous bone marrow contribute to relapse. In total twelve patients were transplanted, four of which have relapsed so far. In three patients, the malignant cells contained the marker gene, whereas the fourth patient was uninformative, as his blasts did not have a leukemia-specific marker. This implies that for an optimal therapeutic response to autologous bone marrow transplantation, it is in these cases a prerequisite that the transplant be depleted of residual tumor cells and hence purging techniques should be considered before transplantation is performed.

In my opinion, the current attitude towards purging is changing and in the near future more attention will be paid to the issue of purging in general and to a

comprehensive evaluation of current and developing alternative purging modalities in particular.

9.4 Future experiments

To achieve clinical implementation of hyperthermia as a purging modality, heat induced cytotoxicity on the normal and leukemic human hematopoietic stem cell compartment has to be investigated thoroughly. In order to increase the therapeutic gain factor of purging, we will try to selectively protect the normal subsets by combining the heat treatment with Goralatide and/or selectively sensitize the leukemic cells by addition of the alkyl-lysophospholipid ET-18-OCH_3 (Edelfosine) during heat treatment. To obtain conclusive insight into the effect of hyperthermia on the primitive leukemic stem cell responsible for the development of leukemia, we will transplant *in vitro* heat treated acute myeloid leukemic cells into SCID mice. These mice will subsequently be analysed for the presence of human leukemic cells (FACS or/and PCR analysis). In addition, post-remission bone marrow with increasing number of leukemic cells will be transplanted to mimic the situation of minimal residual disease. Evaluation of the hyperthermic purging protocol in the clinical situation requires gene marking of the autologous graft. Therefore, experiments are planned to determine the transfection efficiency of a recently developed gene marking system which might be useful to monitor the origin of recurrence at the time of relapse after autologous stem cell transplantation.